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Biochimica et Biophysica Acta 1778 (2008) 1283–1291
Biochimica et Biophysica Acta
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Localization of the Sodium-Taurocholate cotransporting polypeptide in membrane rafts and modulation of its activity by cholesterol in vitro

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Received 2 October 2007; received in revised form 25 January 2008; accepted 28 January 2008
Available online 8 February 2008

Abstract

Background: The relevance of discrete localization of hepatobiliary transporters in specific membrane microdomains is not well known. *Aim:* To determine whether the Na^+/taurocholate cotransporting polypeptide (Ntcp), the main hepatic sinusoidal bile salt transporter, is localized in specific membrane microdomains. *Methods:* Presence of Ntcp in membrane rafts obtained from mouse liver was studied by immunoblotting and immunofluorescence. HEK-293 cells stably transfected with rat Ntcp were used for *in vitro* studies. Expression, localization and function of Ntcp in these cells were assessed by immunoblotting, immunofluorescence and biotinylation studies and Na^+-dependent taurocholate uptake assays, respectively. The effect of cholesterol depletion/repletion assays on Ntcp function was also investigated. *Results:* Ntcp localized primarily to membrane rafts in *in vivo* studies and localized partially in membrane rafts in transfected HEK-293 cells. In these cells, membrane cholesterol depletion resulted in a shift of Ntcp localization into non-membrane rafts, which correlated with a 2.5-fold increase in taurocholate transport. Cholesterol repletion shifted back part of Ntcp into membrane rafts, and normalized taurocholate transport to values similar to control cells. *Conclusion:* Ntcp localizes in membrane rafts and its localization and function are regulated by membrane cholesterol content. This may serve as a novel regulatory mechanism of bile salt transport in liver.

Keywords: Bile acid; Caveolin; Lipid microdomain; Lipid raft; Membrane transport

Transport of bile salts (BS) by the hepatocytes is a vital function of the liver since these amphipathic molecules are the main driving force for bile secretion [1,2]. Handling of BS by liver cells involves several steps: a) sinusoidal uptake from portal blood by the *S*odium-*T*aurocholate *C*otransporting *P*olypeptide (Ntcp, Slc10a1) and members of the organic anion transporting polypeptides (OATP's) family, b) transcellular translocation to the canalicular membrane and c) active secretion into bile by the bile salt export pump (BSEP, Abcb11) [3]. Regulatory mechanisms of BS transport have been described for all these steps and include processes at both transcriptional and post-transcriptional levels. The majority of the published data on this topic concern to transcriptional control of hepatobiliary transporter genes and come from experimental models for pathophysiological conditions such as cholestasis [1,3]. Available data on post-transcriptional regulation of BS transporters are less abundant [4–6]. Several mechanisms have been described as being involved in this type of regulation including covalent modifications (e.g., phosphorylation), substrate competition, and subcellular localization related to rapid endo- or exocytosis of transporter-bearing vesicles into and from the plasma membrane [6]. However, additional molecular mechanisms may exist and need to be characterized. Recent work has shown that the association of certain hepatic transporters (e.g. aquaporins and the ABC transporters *P*-Glycoprotein/Abcb1 and Breast Cancer Resistant Protein, BCRP/Abcg2) to specific cholesterol/sphingolipids-rich membrane domains may have functional consequences [7–9].

Abbreviations: BS, bile salts; BSEP, bile salt export pump; Cav-1, caveolin-1; MR, membrane rafts; HEK 293, Human embryonic kidney 293; MCD, Methyl-β-Cyclodextrin; Ntcp, *S*odium-*T*aurocholate *C*otransporting *P*olypeptide

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doi:10.1016/j.bbamem.2008.01.020

Although the existence of these membrane domains in (generically named membrane rafts [10]) has been matter of controversy [11] the concept that membrane rafts are implicated in a variety of cell functions including signal transduction, protein sorting in polarized cells, apoptosis and pathogen entry among others, is now widely accepted [12,13]. Most of the information on this topic has been generated using two simple methods, the analysis of detergent resistance of membranes and cholesterol depletion [12]. Thus, presence of a given protein in detergent-resistant membranes and the fact that cholesterol modulators affect its function in cells is consistent with a role for rafts [12,14,15].

Data on membrane compartmentalization of BS transporters and its potential functional importance is emerging. For example, a recent paper by Annaba et al. [16] have shown that the ileal bile acid transporter (ASBT, Slc10a2) is associated with lipid rafts which have functional implications. Thus, the present study was conducted to explore if Ntcp, the main sinusoidal hepatic BS transporter [17] which has structural similarities with ASBT, is also localized in membrane rafts in the liver and how this discrete localization may influence its transport activity. Our results indicate that in *ex-vivo* livers Ntcp localizes predominantly in membrane rafts and that change in this localization in transfected cells are associated with reciprocal changes in the function of this BS transporter.

1. Materials and methods

1.1. Reagents, animals and cell lines

Unless otherwise stated, reagents were purchased from Sigma. C57BL6 mice (15–20 g body weight) were housed in transparent polycarbonate cages, with wood chip bedding at a 12 h light/darkness cycle, a temperature of 21 °C, and a relative humidity of 50% throughout the acclimatization (at least 1 week) period and permitted *ad libitum* consumption of water. Animal experiments were approved by the Local Ethics Review Committee on Animal Experiments according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86–23 revised 1985). Human embryonic kidney (HEK) 293 cell line (ATCC CRL 1573) that was purchased from American Type Culture Collection (Manassas, VA) was used in cell culture experiments.

1.2. Transfection of HEK293 cells with rat Ntcp

HEK-293 were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.5 μg/ml fungizone at 37 °C with 5% de CO_2 and 95% humidity. Stable transfection with rat Ntcp [18] was achieved using a pcDNA 3.1-Ntcp expression vector containing the full-length rat *ntcp* cDNA (GenBank accession number NM_017047) using the Lipofectamine™ Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's suggested protocol. Transfected cell lines were selected by growth with 800 μg/ml antibiotic G418 Sulfate and maintained with 400 μg/ml of the antibiotic G418 Sulfate (Geneticin) (Life Technologies, Invitrogen, Carlsbad, CA). Ntcp transport function in HEK-293-Ntcp cells was assessed by determining Na^+-dependent taurocholate uptake as previously described by us [19]. In these experiments choline was used as sodium replacement and taurocholate uptake was calculated as the difference between Na^+-containing and Na^+-free buffers. Results were expressed as picomoles of TC per milligram of protein. In selected experiments, HEK-293 cells were infected with replication-defective adenoviral vectors carrying the full-length human caveolin-1 (Ad.Cav-1) as previously described by our laboratory [20].

1.3. Confocal immunomicroscopy

Ntcp immunolocalization studies in mouse livers were performed as described previously [20]. Briefly, livers were perfused via the portal vein with cold phosphate-buffered saline and then excised and frozen under isopentane in liquid nitrogen. Qualitative distribution of Ntcp and caveolin-1 was assessed by indirect immunofluorescence using monoclonal anti-Cav-1 (clone 2297, dilution 1:100; Transduction Laboratories, Cincinnati, OH) or anti-Ntcp [19]. Labeled sections were examined on a Zeiss Axionplan microscope, and digital images were processed using Axionvision imaging software (Carl Zeiss, Göttingen, Germany). For cell culture experiments, indirect immunofluorescence microscopy was performed on a confluent monolayer of transfected cells. The cells were fixed with Paraformaldehyde and permeabilized with 0.2% v/v Triton X-100. Free aldehyde groups were blocked with 50 mM NH_4Cl. Nonspecific sites were blocked with Gelatin Type Bloon 0.2% w/v diluted in PBS. Incubation with primary antibodies for rat Ntcp, transferrin receptor (Tf-R) and caveolin-1 (Cav-1) was carried out for 2 h at 37 °C in a humid chamber. After washing with PBS 5 times for 5 min, cells were incubated with secondary antibodies conjugated to FITC and rhodamine for 1 h, washed with 1× PBS and mounted in Fluoromount G (Electron Microscopy Sciences, Hatfield, PA) for confocal microscope observation.

1.4. Isolation of membrane lipid microdomains

Membrane rafts were isolated using a flotation assay previously described by Mora and coworkers [21]. Briefly, 200 mg of mice liver tissue was homogenized in 1 ml of MBS buffer (MES 25 mM pH 6.5, NaCl 150 mM and PMSF 1 mM) containing Triton X-100 (1% v/v) using a loose-fitting Dounce homogenizer (10 strokes on ice). Extraction was performed on ice for 30 min, and sucrose concentration in samples was adjusted to 40% using MBS containing 65% sucrose w/w, overlaid with 1.3 ml of 30% and 1.3 ml of 5% sucrose/MBS and centrifuged for 16–20 h at 200,000 ×*g* at 4 °C in a TST 60.4 rotor (Sorvall, Asheville, NC). Membrane rafts were visible as an opalescent band at the 30%/5% interface of the sucrose gradient. The total volume of the gradient was fractionated into twelve tubes beginning with the top of the gradient.

Membrane raft isolation from HEK-293-Ntcp cells was carried out from three confluent 150 mm dishes after washing with PBS. Cells were pelleted by centrifugation at 80 ×*g* for 10 min and further scraped in 500 μl of MBS buffer containing 1% Triton X-100 (v/v). The cells were homogenized in ice by 20 passages through a 25-gauge needle. After this step, the protocol for membrane raft isolation was the same as that used for mouse liver tissue as described above.

1.5. Immunoisolation of caveolae from total hepatic lipid rafts

A procedure described by Stan et al. [22] with few modifications was used. The membrane raft fractions isolated from the liver using Triton X-100 1% (3–5 fractions) was diluted in 10 ml of MBS buffer and centrifuged at 100,000 ×*g* for 1 h to 4 °C in rotor T-875, the pellet obtained was resuspended in 1 ml of TNE buffer (Tris–HCl 25 mM pH 7.5, NaCl 150 mM, EDTA 5 mM and PMSF 1 mM). The samples were pre-cleared in 50 μl of Protein-A Sepharose for 1 h to 4 °C and centrifuged at 280 ×*g* for 1 min. The pre-cleared samples were mixed with 2 μg of monoclonal IgG_{2a} anti Caveolin-1 (clone 2234, Transduction Labs) and incubated for 1 h at 4 °C with constant rocking. The immunocomplexes were collected with 50 μl of Protein-A Sepharose by incubation for 1 h at 4 °C. The samples were centrifuged at 280 ×*g* and the supernatant was recovered by pelleting to 100,000 ×*g* and stored in ice. The immunoprecipitates were washed three times with TNE buffer and eluted by addition of 50 μl of Laemmli buffer and loaded onto SDS-PAGE, after heating for 10 min at 37 °C and centrifugation at 5000 ×*g*. The supernatant containing caveolae domains was loaded on SDS-PAGE together with the same volume of non-caveolar membrane rafts and caveolae-containing membrane raft samples used at the beginning of the protocol.

1.6. Extraction of lipid raft with different detergents

For these experiments the membrane rafts fractions of the sucrose gradient (fractions 3 to 5) were diluted in 8 ml of TNE buffer (Tris–HCl 25 mM pH 7.5, NaCl 150 mM, EDTA 5 mM and PMSF 1 mM) and centrifuged at 100,000 ×*g* in a T-875 rotor for 1 h at 4 °C. The pellet containing membrane rafts were

resuspended in 1 ml of TNE with a 25G needle and syringe and re-extracted for 30 min on ice with different detergents for each treatment (Chaps 20 mM, Octy-β-glucosyde 60 mM, Deoxycholate 9 mM, SDS 1% as described previously [23]. Lysates were centrifuged at 100,000 ×*g* for 1 h at 4 °C. The insoluble fraction contained in the pellet was resuspended in 1 ml of TNE with the soluble fraction remaining in the supernatant. Soluble and insoluble fractions were subjected to immunoblot analysis as described below.

1.7. Treatment of Cells with Methyl-β-Cyclodextrin (MCD) and cholesterol-MCD complexes

All experiments using cells were performed in serum-free DMEM as described by Luker et al. [24]. In cholesterol depletion assays, cells were incubated in 10 mM of MCD for 30 min to 37 °C. For cholesterol repletion assays, cells were first treated with MCD, washed in PBS and incubated with 0.2 mM Cholesterol-MCD complex previously prepared as described by Klein et al. [25] for 1 h to 37 °C. Control cells were incubated with medium alone. After each treatment cells were washed three times with PBS and used for experiments to measure taurocholate transport or isolation of membrane rafts following immunoblot analysis.

Cell surface Biotinylation. These experiments were performed using sulfo-NHSSS-biotin (0.5 mg/ml; Pierce, Rockford, IL) in an appropriate buffer (PBS/CaMg (100 μM $CaCl_2$ and 1 mM $MgCl_2$), as previously described [26], with labeling for 60 min under constant shaking at 4 °C to stop endocytosis and internalization of antigens. The reaction was stopped adding 100 mM glycine in PBS/CaMg for 10 m at 4 °C. After washing with PBS pH 7.4, cells were scraped and centrifuged at 1000 rpm for 2 min and homogenized in 500 μl of TNE buffer (Tris–HCl 25 mM pH 7.6, EDTA 5 mM, NaCl 150 mM, Triton X-100 1% v/v in presence of protease inhibitors). After 30 min in ice, membrane microdomains were isolated using the flotation assay described above. 50 μl aliquots from fractions 3–5 (rafts) and fractions 9–12 (non-rafts) were diluted to 1 ml of TNE buffer and used for immunoprecipitation of biotinylated antigens with streptavidin-agarose. Immunoprecipitates were then subjected to SDS-PAGE and blots were probed for Ntcp. To assess non-surface Ntcp content, 500 μl of supernatants were precipitated with TCA and 25 μl of sample used for western blot experiments.

1.8. Immunoblotting

Samples were mixed with loading buffer of SDS-PAGE (5X) and heated for 5 min to 100 °C, except for immunoblotting of Ntcp. Proteins separated by the gel were transferred into nitrocellulose membranes using Trans-blot system (Bio-Rad Laboratories, Hercules, CA). Membranes were washed in TBS-T (Tris–HCl 25 mM pH 7.5, NaCl 150 mM and Tween-20 0.05% v/v) and blocked with 5% w/v of non-fat dry milk dissolved in TBS-T overnight to 4 °C. After rinsing with TBS-T, membranes were incubated for 1.5 h with primary antibodies in blocking buffer at room temperature. After this step, membranes were rinsed three times and incubated for 1 h with secondary antibodies conjugated to HRP. Following washing with TBS-T, blots of immunoreactive complexes were visualized using the ECL™ Western Blotting Detection System (Amersham Biosciences (GE Healthcare, Piscataway, NJ).

1.9. Isolation of total membranes from HEK-293 cells

Confluent HEK-293 cells from one 100 mM dish were transfected with Ntcp and wild-type cells were scraped in PBS and pelleted by centrifugation at 1,000 *g* The cell pellet was resuspended in 1 ml of 25 mM Tris–HCl pH 7.5, 2 mM $MgCl_2$, 250 mM sucrose and 1 mM, PMSF, homogenized with 20 times on ice using tight-dounce and centrifuged for 10 min at 1000 ×*g* at 4 °C. The supernatant was pelleted to 100,000 ×*g* for 1 h at 4 °C, the pellet containing total membranes was resuspended in the homogenization buffer by 10 passages in ice through a 25G needle. Protein content in this fraction was measured by Bradford method using BSA as standard and stored at −80 °C.

1.10. Lipid extraction and free cholesterol measurements

Lipid extraction was carried out using 200 μl of each fraction of the gradient or pooled fractions of membrane rafts and non- membrane rafts dissolved in

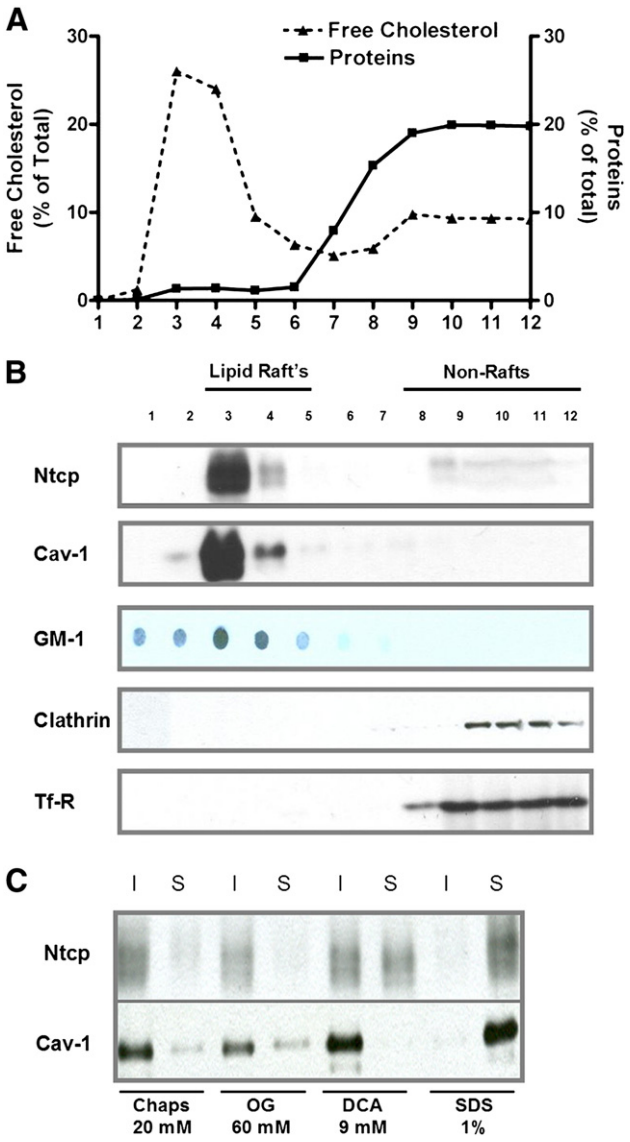

Fig. 1. A: Cholesterol and protein content of fractions obtained from mouse liver after Triton X-100 treatment and centrifugation in a sucrose gradient as described in materials and methods. The Triton-insoluble, lipid-enriched microdomain (lipid rafts) band (fractions 3 to 5) was located at the 5%/30% sucrose interface. B: Representative immunoblot analysis of obtained fractions (numbers on the top). The *S*odium-*T*aurocholate *C*otransporting *P*olypeptide (Ntcp) was detected mainly in fractions 3–4 that also contain both caveolin-1 (Cav-1) and glycosphingolipid GM1, known markers for lipid rafts. Clathrin and transferrin receptor (Tf-R) were localized to the bottom of the gradient (fractions 8 to 12) in the Triton-soluble fractions indicating that they reside in non-raft microdomains. C: Representative western blots of Ntcp and Cav-1 using lipid rafts (fractions 3 to 5) treated with different detergents. I (insoluble) and S (soluble) indicate the two fractions obtained after treatment. Chaps and octyl-β-D-glucoside (OG) only marginally removes Cav-1 but does not remove Ntcp. In contrast, deoxycholic acid (DCA) removed about half of the protein mass of Ntcp but does not remove Cav-1. Treatment with Sodium dodecyl sulphate (SDS) removes both proteins from the cholesterol-rich, detergent-resistant fractions. The different effects of detergent treatment suggest that Ntcp and Cav-1 localize to different lipid microdomains. Alternatively, the observed differences in this extraction experiments could reflect the differences in the membrane attachment of Ntcp and Cav-1 (see the text).

300 µl of methanol. Cholesterol was separated by Folch method with 600 µl chloroform, vortexed and incubated for 2 h at 4 °C and lipid phase was separated by centrifugation at 1000 ×*g* for 10 min. The hydrophobic phase was obtained and dried using N_2. Lipids were resuspended in 100 mM Tris–HCl pH 7.6, 5 mM phenol, aminoantiypirine and 0.2% v/v Triton X-100 in a bath sonicator. After adding cholesterol oxidase and peroxidase to these samples, free cholesterol content was measured at 500 nm using a standard technique.

1.11. Statistics

All results are expressed as mean±SE. A two-tailed non-paired Student's *t*-test was used to compare differences between groups when appropriate. Values were considered significantly different when the *P* value was equal to or less than 0.05.

2. Results

2.1. Ntcp localizes to membrane rafts of hepatic tissue

Triton X-100 lysates from mouse livers were separated on discontinuous sucrose gradients and then characterized by immunoblotting with antibodies against specific proteins including Ntcp. Twelve fractions (1 ml each) were collected from the bottom of the gradient (fraction 1) to the top of the gradient (fraction 12). The Triton-insoluble, lipid-enriched microdomain band (fractions 3 and 5) was located at the 5%/30% sucrose interface. Of note, only a small proportion of total proteins are recovered in these fractions while more than 50% of proteins were detected in the low-cholesterol fractions (Fig. 1A). As shown in Fig. 1B, immunoblot analysis of the different fractions revealed that most of the protein mass of Ntcp was found in fractions 3–4 of the gradient that also contain both Cav-1 and glycosphingolipid GM1, which are positive markers of membrane rafts. As expected, clathrin and Tf-R were localized to the bottom of the gradient with Triton-soluble fractions in agreement with previous reports showing that these proteins are not associated with membrane rafts. Further studies using additional treatment of the isolated membrane rafts with different detergents showed that membrane rafts containing Ntcp differed from those of Cav-1 in its insolubility/solubility properties. As shown in Fig. 1C, deoxycholic acid (DCA) was able to remove about half of the protein mass of Ntcp but did not remove Cav-1 suggesting that these proteins probably localize to different lipid microdomains. Furthermore, treatment with the ionic detergent SDS removes both proteins from the cholesterol-rich, detergent-resistant fractions as previously described for other proteins tightly-associated with membrane rafts [23]. Of note, the above described experiments were also carried out with plasma membrane fractions (data not shown) yielding similar results. An alternate explanation to the observed differences in this extraction experiments, is that the differences in the membrane attachment of Ntcp and Cav-1, the former being a polytopic integral membrane protein [17] and the latter being a non-polytopic membrane protein that anchors to the membrane in a hairpin configuration and interacts with itself to form homo-oligomers [27,28], which determines its susceptibility to removal by detergents.

Immunoisolation studies with specific antibodies for Cav-1 (Fig. 2A) revealed that Ntcp does not co-isolate with this protein, which is in agreement with confocal immunofluorescence studies in mouse liver (Fig. 2B). Collectively, these results suggest that Ntcp isolated with membrane rafts is predominantly

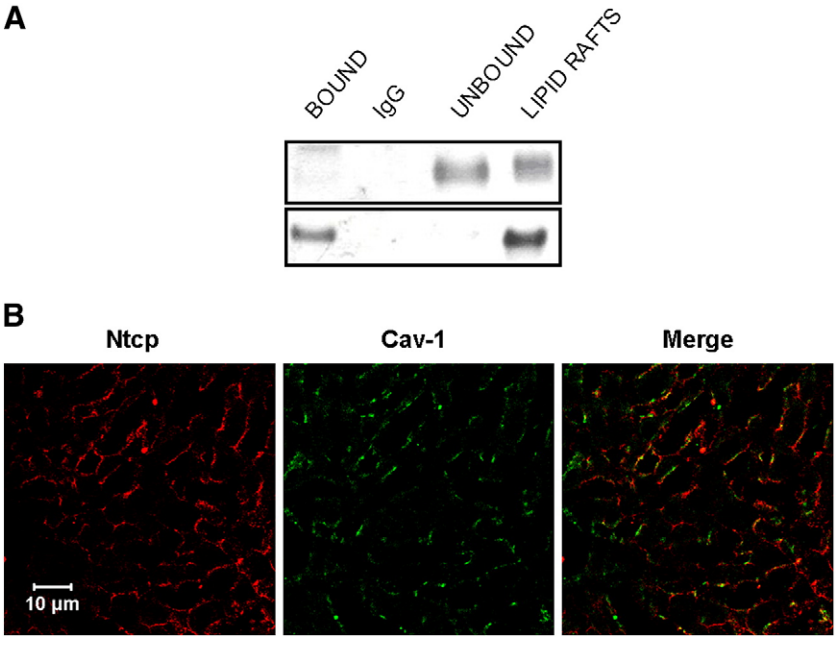

Fig. 2. The *S*odium-*T*aurocholate *C*otransporting *P*olypeptide (Ntcp) and Caveolin-1 (Cav-1) do not colocalize in mouse liver. A: After immunoisolation of Cav-1 from Lipid rafts fractions obtained from mouse liver, equal volumes of bound unbound and total Lipid rafts fractions were loaded and separated by SDS-PAGE. Ntcp was found only in the unbound and total fractions suggesting that this transporter is predominantly restricted to non-caveolar rafts. B: Immunolocalization of Cav-1 and Ntcp in wild-type mouse liver. Livers from normal mice were stained for either Ntcp (left panel) or Cav-1 (middle panel). Bound primary antibodies were visualized with Alexa Fluor 488- (Molecular Probes, Inc., Eugene, OR) or Texas Red- (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD) conjugated secondary antibodies diluted at 1:400 and 1:100, respectively. A merged image is shown in the right panel.

restricted to non-caveolar rafts present in a low-buoyant-density membrane fraction.

2.2. Assessment of Ntcp expression, function and localization in HEK-293 cells

As shown in Fig. 3A, HEK-293 cells stably transfected with the full-length cDNA from rat Ntcp (HEK-293-Ntcp) expressed this protein at similar levels to those observed in rat liver. Functionality of the transporter was demonstrated by the presence of Na^+-dependent taurocholate uptake in HEK-293-Ntcp cells [29.02±3 pmol min^{-1} mg $protein^{-1}$ transfected cells vs. 7.29±0.72 pmol min^{-1} mg $protein^{-1}$ in non-transfected cells, (n=3 each group, P<0.05)]. Localization of Ntcp in plasma membrane was assessed using double immunofluorescence labeling studies with antibodies against Ntcp and Cav-1. As shown in Fig. 3B, Ntcp and Cav-1 do not colocalize in the plasma membrane of HEK-293 cells expressing both cDNAs. In addition, after isolation of membrane rafts from HEK-293-Ntcp cells immunoblot analysis for Ntcp, Tf-R and Cav-1 was carried out to establish if Ntcp localizes to membrane rafts in HEK-293-Ntcp cells. In these studies, Ntcp was detected in both membrane rafts as well as non-rafts fractions (Fig. 4A). These results are consistent with partial localization of Ntcp in non-caveolar membrane rafts in these cells similar to that seen in mouse liver.

2.3. Effects of cholesterol depletion and repletion on Ntcp localization and function in Ntcp-transfected HEK-293 cells

In order to assess if the association of Ntcp with membrane rafts in liver tissue might have functional significance, we investigated the effect of cholesterol depletion and repletion on both localization of Ntcp in membrane rafts and sodium-dependent taurocholate uptake in HEK-293-Ntcp cells. For that purpose, cells were treated with either MCD or Cholesterol-MCD complexes. As shown in Fig. 4A, MCD treatment was associated with an 80% decrease in free cholesterol in membrane preparations of HEK-293 cells while cholesterol re-loading with Cholesterol-MCD complexes normalized cholesterol content. These changes in cholesterol content were associated with modifications in Ntcp

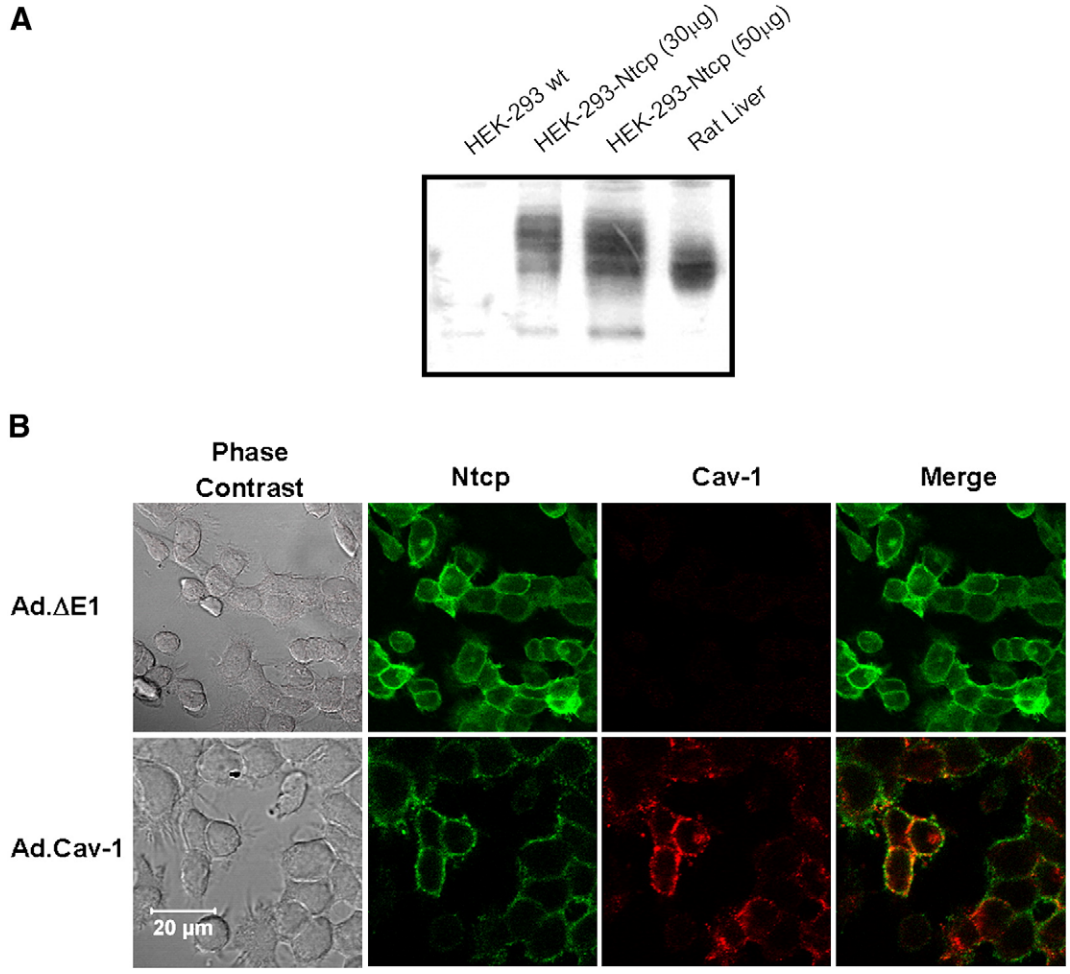

Fig. 3. Detection of the *S*odium-*T*aurocholate *C*otransporting *P*olypeptide (Ntcp) in Ntcp-transfected HEK-293 cells. A: Representative western blot showing Ntcp immunodetection of in wild-type (WT) HEK-293 and Ntcp-transfected HEK-293 cells. A sample of a normal rat liver was used for comparison. B: Confocal immunofluorescence of Ntcp-transfected HEK-293 cells infected with an E1- and E3-deleted replication-defective adenoviral vectors carrying the full-length human caveolin-1 (Ad.Cav-1). Control adenovirus (Ad.ΔE1) contained the same E1 and E3 deletions without the transgene expression cassette. Merged image shown in the far right indicates a distinct localization of Ntcp and Cav-1 in doubly-transfected cells.

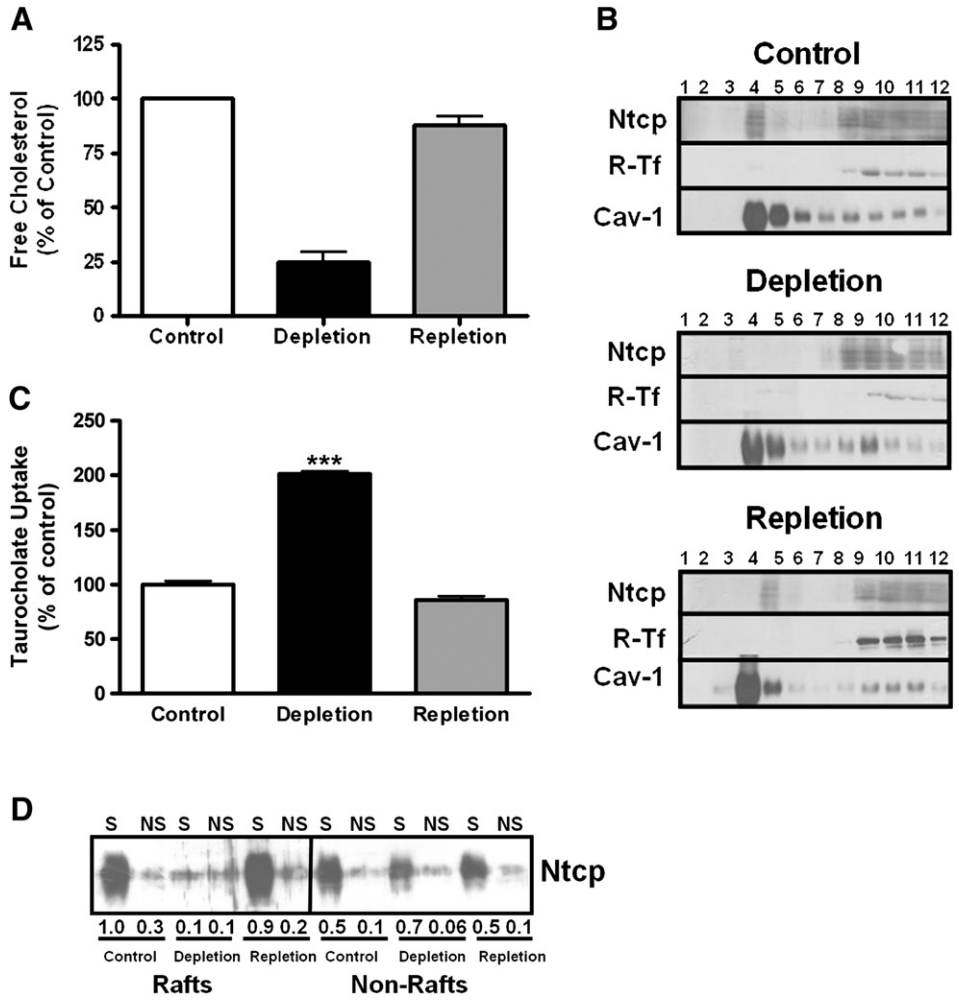

Fig. 4. Effects of cholesterol depletion and repletion on membrane cholesterol content, Ntcp membrane localization and function in Ntcp-transfected HEK-293 cells. A: MCD treatment was associated with an 80% decrease in free cholesterol in membrane preparations of HEK-293 cells while Cholesterol-MCD treatment normalized cholesterol content in depleted cells. B: Representative western blots of the sodium-taurocholate cotransporting polypeptide (Ntcp), Cav-1 and transferrin receptor (Tf-R) using fractions obtained from HEK-293-Ntcp cells treated with MCD and Cholesterol-MCD complexes after Triton X-100 treatment and centrifugation in a sucrose gradient, as described in materials and methods. Ntcp abundance varies within membrane domains. While this transporter was localized to both lipid rafts and non-rafts fractions in control cells, cholesterol depletion after MCD treatment determined a shift of Ntcp into non-rafts fractions (fractions 8–12), which is reversed by cholesterol repletion (re-appearance of Ntcp in fraction 4). The impact of cholesterol alteration on Tf-R and Cav-1 localization was negligible since presence of these proteins in non-rafts and lipid rafts fractions respectively remained unaffected by cholesterol manipulations. C: Changes in sodium-dependent uptake of taurocholate in Ntcp-transfected HEK-293 after cholesterol depletion and repletion. A significant increase in the taurocholate uptake was observed after cholesterol depletion induced by MCD treatment. Restoration of cellular cholesterol by the addition of cholesterol-MCD complexes to the depleted cells led to restoration of the transport function of Ntcp. D: Representative western blot showing the effects of cholesterol depletion/repletion on surface localization of Ntcp in HEK-293-Ntcp cells. HEK-293-Ntcp cells were subjected to biotinylation at 4 °C using sulfo-NHS-SS-biotin, lysed and surface biotinylated proteins were precipitated with streptavidin-agarose from equal amounts of total cellular protein. Precipitated proteins (surface, S) and proteins in the supernatant (non-surface, NS) were separated on SDS-polyacrylamide gel electrophoresis and electroblotted to nitrocellulose blots. Western blotting analysis was performed with anti-Ntcp antibody. The relative abundance of Ntcp in the S and NS fractions obtained from control and treated cells (subjected to cholesterol depletion/repletion) is shown in the Figure. Numbers refers to the ratio between the densitometric value of the respective band and that from the control cells. Results displayed in graphs are presented as % of control values (for either cholesterol content or taurocholate uptake) and represent means ± SE obtained from at least three separate experiments. $*P < 0.05$ or less compared to control.

localization within membrane domains as shown in Fig. 4B. In fact, Ntcp was localized to both membrane rafts and non-rafts fractions in control cells (Fig. 4B, upper panel) and cholesterol depletion resulted in a shift of Ntcp into non-rafts fractions (Fig. 4B middle panel), which was partially reversed by cholesterol repletion (Fig. 4B lower panel). The impact of changes in plasma membrane cholesterol content on Tf-R and Cav-1 localization was negligible since presence of these proteins in membrane rafts and non-rafts fractions respectively remained unaffected after treatments with either MCD or cholesterol-MCD complexes. These data indicate that these maneuvers affect mainly fractions of membrane rafts containing Ntcp and that Cav-1-containing membrane rafts or caveolae are highly resistant to cholesterol depletion by MCD. Interestingly, changes in plasma membrane cholesterol content of HEK-293-Ntcp cells also correlated with significant changes in their sodium-dependent uptake of taurocholate. Of note, as shown in Fig. 4C, cholesterol depletion of HEK-293-Ntcp cells led to a significant

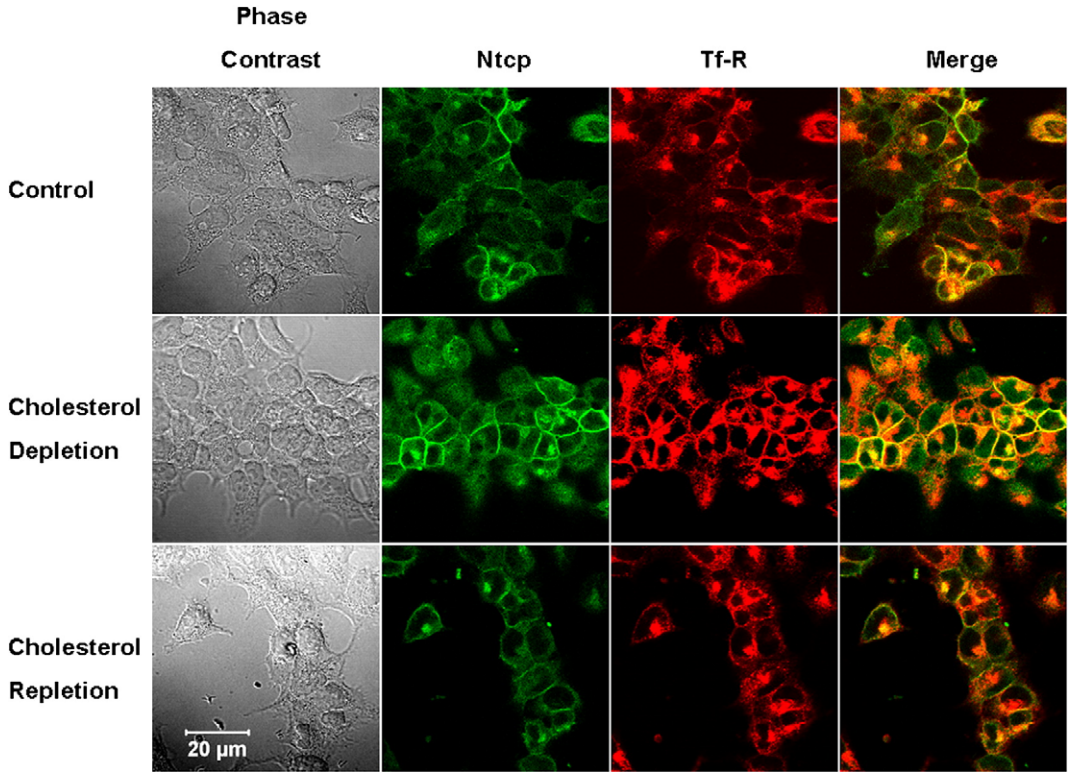

Fig. 5. Immunolocalization of Ntcp and Tf-R in Ntcp-transfected HEK-293 cells subjected to membrane cholesterol content manipulations. Under basal conditions, Ntcp and Tf-R localized to the plasma membrane and also in intracellular compartments, with discrete co-localization only at the plasma membrane in some cells, particularly those with higher expression levels. Cholesterol depleted cells showed an intensification of the signal for both proteins at the plasma membrane, with co-localization of Ntcp and Tf-R at the cell surface but not intracellularly. Cholesterol repletion decreases the signal of both membrane proteins at the cell surface due to an apparent internalization and dissociation of both proteins in intracellular compartments.

2-fold increase in the sodium-dependent uptake of taurocholate and restoration of cellular cholesterol, by the addition of cholesterol-MCD complexes to the depleted cells, restored transport function of Ntcp to values similar to those seen in control cells (Fig. 4C). Membrane biotinylation studies (Fig. 4D) showed that the observed increase in transport activity after cholesterol depletion was accompanied by a redistribution of Ntcp protein between different membrane microdomains (rafts and non-rafts fractions) with no significant changes in the intracellular Ntcp content.

Finally, in confocal immunomicroscopy studies (Fig. 5) we observed that cholesterol depleted cells exhibited a stronger plasma membrane signal and partial co-localization of Ntcp and Tf-R which was reversed after cholesterol repletion due to the apparent internalization of both proteins (Fig. 5).

3. Discussion

The aim of this study was to determine whether Ntcp, the main BS importer of the hepatocyte, is localized in membrane microdomains of hepatocytes and if this localization influences its function. Our results indicate that Ntcp does localize mainly in detergent-resistant fractions from the mouse liver (Fig. 1B), namely membrane rafts [10]. Moreover, using Ntcp-transfected cells, we found that changes Ntcp localization in the membrane modifies sodium-dependent BS transport thus providing a novel mechanism of regulation for this transporter.

The presence of membrane rafts in the plasma membrane of hepatocytes has been shown by several authors [7,29,30]. As observed in other cell types, some membrane proteins are preferentially localized in detergent-resistant fraction domains [13]. For example, work from Mazzone et al. indicate that the water channel aquaporin 8 is localized in these domains in addition to other proteins involved in bile formation namely Anion exchanger 2 and multidrug resistant-associated protein 2 (Abcc2) [7]. Using similar techniques, we found that Ntcp is also localized in membrane rafts in the plasma membrane of mouse hepatocytes. Interestingly, this observation is in agreement with findings of a recent study that characterized the membrane rafts proteome using capillary liquid chromatography-tandem mass spectrometry and identified Ntcp as one of the 196 proteins associated with membrane rafts fractions isolated from rat liver [31]. In our experiments, we confirmed that Ntcp localizes in specific membrane microdomains of mouse hepatocytes and further characterized the association between Ntcp and membrane rafts treating these fractions with different detergents and performing immunoisolation studies as well as confocal immunofluorescence imaging. Collectively, our results indicate that Ntcp is preferentially localized in membrane rafts different from those where caveolin is localized as is the case for other proteins such as the epidermal growth factor (EGF) receptor, phosphatidylinositol 4-kinase, phosphoinositides and GPI-linked proteins among others [32].

Association of membrane proteins with membrane rafts has been related to a number of cellular processes including protein

sorting, signaling and protein functionality [12,33,34]. In the case of membrane transporters, it has been shown that residency in these membrane microdomains might be important for the transport capacity as it has been described for the canalicular ABC transporters *P*-glycoprotein/Abcb1 [8] and BCRP/Abcg2 [9], the HDL receptor, SR-BI [35] and more recently for the ileal bile acid transporter ASBT, a symporter that belongs to the same family as Ntcp [16,17]. Therefore, we wanted to study the effects of altering the cholesterol concentration of the plasma membrane, which should result in a shift of a substantial proportion of Ntcp from membrane rafts to the detergent-soluble membranes, and on Ntcp function. For these purposes, we stably transfected with the full-length cDNA for rat Ntcp into HEK-293 cells and assessed the sodium-dependent BS transport using conventional techniques. Localizaion of Ntcp in transfected cells was somewhat different to that observed in the mouse liver with only part of Ntcp being found in membrane rafts which may be due to different plasma membrane lipid composition or due to overexpression of the transporter. However, as intended in these experiments, changes of the cholesterol concentrations in these cells did result in a shift of Ntcp from detergent-insoluble membranes to detergent-soluble parts of the membrane and vice versa. Thus, while the shift of a substantial portion of Ntcp to detergent-soluble parts of the membrane in HEK-293-Ntcp was accompanied by a two-fold increase in sodium-dependent BS uptake, shifting back Ntcp to detergent-insoluble parts of the membrane was associated with normalization of BS uptake. These findings strongly suggest that changes in the lipid environment in which Ntcp is located modulates its function. One possible explanation for this phenomenon could be due to changes in plasma membrane fluidity. Enhancement or reduction of transporter activity in response to changes in plasma membrane fluidity has indeed been proposed as important events in regulating BS transport in both sinusoidal and canalicular domains of hepatocyte membrane [36,37]. Restoration of Ntcp transport function to control values after increasing cellular cholesterol content by the addition of cholesterol-MCD complexes to the depleted cells is consistent with this concept. However, changes in membrane fluidity may not be the only explanation for changes in transporter function since it has been shown that decreasing cellular cholesterol and disruption of membrane rafts do not affect transporter function uniformly. In fact, the function of some hepatic transporters decreased when translocated out of membrane rafts as is the case of *P*-Glycoprotein [8,38] and ASBT [16]. Thus, additional mechanisms should be considered. Since it has been found that, in addition to plasma membrane, Ntcp also localizes to an intracellular pool that serves as a reservoir from which they can be recruited to the plasma membrane in response to an increased transport demand (e.g. post-prandial period) [39–41], it is also possible that depletion of cholesterol may induce changes in the rafficking of Ntcp, to the cell surface. In our confocal immunomicroscopy studies (Fig. 5) we did observe that cholesterol depleted cells exhibited a stronger plasma membrane signal for Ntcp which was reversed after cholesterol repletion due to the apparent internalization of the transporters. Thus it could be speculated that cholesterol depletion might promote insertion of Ntcp through activation of this pathway. However, this is not supported by our biotinylation studies. This discrepancy may be related to technical issues and needs further study.

In conclusion, our results indicate that Ntcp localizes predominantly in non-caveolar membrane rafts in the liver and that modifications in its membrane microdomain localization are associated to reciprocal changes in the function of this BS transporter. The importance of these findings for the regulation of BS transport in the liver in both physiological and pathophysiological conditions, such as cholestasis, deserves further study.

Acknowledgements

This work was partially supported by a grant from the Fondo Nacional de Ciencia y Tecnología (FONDECYT # 1040820 to Juan Francisco Miquel and #1050780 to Marco Arrese) and a grant from the National Institutes of Health (Meenakshisundaram Ananthanarayanan HD20632). Dr. Bruno Stieger (Institute of Clinical Pharmacology and Toxicology at the Department of Medicine, University Hospital, Zurich, Switzerland) generously provided anti-Ntcp antibodies.

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